SUPPRESSION OF HYDROGEN EXCHANGE IN STAPHYLOCOCCAL NUCLEASE BY LIGANDS

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In 1954, Hvidt and Linderstrøm-Lang[1] introduced the study of hydrogen exchange in proteins as a probe of secondary and tertiary structure. By comparing the kinetics of exchange of deuterium atoms between protein and water with that of model compounds, they and other workers were able to make inferences about the conformation of polypeptide chains in solution.[2] Subsequent modifications of this method, using tracer amounts of tritium,[3, 4] and gel filtration[4] rather than lyophilization to separate the labeled protein from the aqueous medium, have had practical and theoretical advantages. Differences in the kinetics of hydrogen exchange between enzymes in the presence and in the absence of their prosthetic groups or substrates have been shown in several cases.[5-9] A further modification of these techniques, described in this paper, has allowed us to make a study of the interactions of proteins with various ligands and of polypeptide chains with each other.

The studies reported below were done with an extracellular nuclease produced by *Staphylococcus aureus*. This nuclease cleaves phosphodiester bonds in both DNA and RNA.[10] Its amino acid sequence has been determined,[11] and its physical and enzymatic properties have been studied.[10] Calcium ions have been found to be essential in the binding to this nuclease of substrates or inhibitory substrate analogues, such as deoxythymidine-3',5'-diphosphate (pdTp). Studies involving this inhibitor have suggested that the nucleotides fit into a groove in the molecule that contains several tyrosyl groups, causing the exclusion of water but only a small conformational change. Accompanying these effects is a stabilization of the protein to the action of several proteolytic enzymes. At alkaline pH, in the presence of calcium chloride and pdTp, trypsin digestion produces three polypeptide fragments. The longer two of these, designated Nase-T-p₂ and Nase-T-p₃ (44 and 100 amino acid residues, respectively), associate to give a protein, nuclease-T, that has about 10 per cent of the enzymatic activity of the original nuclease.[12] We have used the measurement of tritium-hydrogen exchange to study the effects of calcium chloride and pdTp binding on nuclease and on the interactions of the Nase-T-p₂ and Nase-T-p₃ fragments.

We have observed significant suppression of tritium exchange after the addition of ligands to tritium-labeled nuclease. This tritium trapping may reflect changes in protein *motility*[13] and the direct shielding of parts of the protein by the ligands, as well as changes in mean conformation. As discussed below, this trapping effect may be analogous to the effect caused by corking a bottle.

Methods.—Tritiated water (100 mc/gm, lot no. 380-15) was obtained from the New England Nuclear Corp. Sephadex G-25 (Pharmacia) was "fine" grade. Deoxythymidine-3',5'-diphosphate was purchased from Calbiochem.

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Proteins: Staphylococcal nuclease (Nase) was isolated from the growth medium of *Staphylococcus aureus* (Foggi strain).[14, 15]

Nuclease-T was prepared by limited digestion of Nase with trypsin (at pH 8.1) in the presence of calcium chloride and pdTp, and was purified by chromatography on phosphorylated cellulose.[12] Nase-T-p$_2$ and Nase-T-p$_3$ were resolved by gel filtration in 50% acetic acid on a Bio-Gel P-20 (3 × 200 cm) column. The Nase-T-p$_2$ and Nase-T-p$_3$, as characterized by amino acid analysis and reconstitution of nuclease activity upon recombination, corresponded to the previously described preparations.[12]

A spectrophotometric Nase assay, using denatured DNA as substrate, was employed.[16]

Labeling: Stock solutions of tritium-labeled Nase were prepared by dissolving 6.0–7.0 mg of lyophilized Nase per ml of 0.05 M Tris-HCl buffer, pH 8.1. Five μl of tritiated water (THO) was added to each milliliter of the Nase solution. Stock solutions of the Nase-T-p$_2$ and Nase-T-p$_3$, at 4 mg/ml and 9 mg/ml, respectively, were prepared with the same buffer and concentration of tritium. The protein solutions were kept for several days at 2°C to allow the establishment of tritium–hydrogen exchange equilibrium.

Gel filtration: In order to separate the labeled protein from the tritiated water, gel filtration on Sephadex G-25 was employed as suggested originally by Englander.[4] We have used columns of 2 × 15 cm, or longer, specially designed for minimal dead space, with flow rates of about 1 ml/min.

The labeled protein solution was equilibrated at room temperature before each experiment. Aliquots of 0.5 ml, to which 0.05 ml of 0.1 M CaCl$_2$ and 0.05 ml of a pdTp solution (3.5 mg/ml) was added, were used. If no ligand solution was added, the sample volume was made to 0.6 ml with the Tris-HCl buffer. With these concentrations, the molar ratio of pdTp to Nase was about 1.5 to 1. In studies of the effects of ligands, the equilibrating buffer contained the indicated concentrations of ligands.

Fractions of 2.0 ml each were collected. As determined by measurements of radioactivity, optical density at 280 mμ, and enzyme activity, complete resolution of the labeled protein from the free tritiated water was achieved with good reproducibility. It was found that the column could be re-used after being washed with several hundred milliliters of water. Recovery of protein from the Sephadex columns usually exceeded 90%. We assumed similar recoveries in experiments with added ligands where the protein absorbancy was masked.

Counting: A 1.0-ml aliquot from each fraction was added to 15 ml of Bray's liquid scintillator.[17] Counting, for periods sufficient to give less than 5% variance, was done in a refrigerated Packard Tri-Carb liquid scintillation spectrometer, model 3214. Counts per minute were converted to disintegrations per minute (dpm) after internal standardization with toluene-H^3.

To determine the quantity of tritium per molecule of Nase, the disintegrations per minute in the excluded peak were summed; in the studies with pdTp the disintegrations per minute in the four tubes (five with the 40-cm column) containing the peak were summed. These values are listed in Table 1. The extent of labeling under any given condition was calculated from these sums, and the specific activity in the labeling mixture, using the formula of Englander.[4]

Results.—Trapping of H^3 by addition of pdTp and calcium chloride: When labeled nuclease is separated from the tritiated water on a Sephadex G-25 column, one observes a peak of radioactivity corresponding to the distribution of the protein, then radioactivity due to back-exchanging tritium atoms, and then a large peak of free THO. Those hydrogens back-exchanging "instantaneously" are obscured by this large peak of free THO.

Figure 1A shows the elution pattern of the gel filtration of 3.0 mg of nuclease on a 2 × 40-cm column of Sephadex G-25. The peak of radioactivity associated with the protein corresponded to about five tritium atoms per molecule of protein, as shown in Table 1. The pattern shows that the major part of the tritium
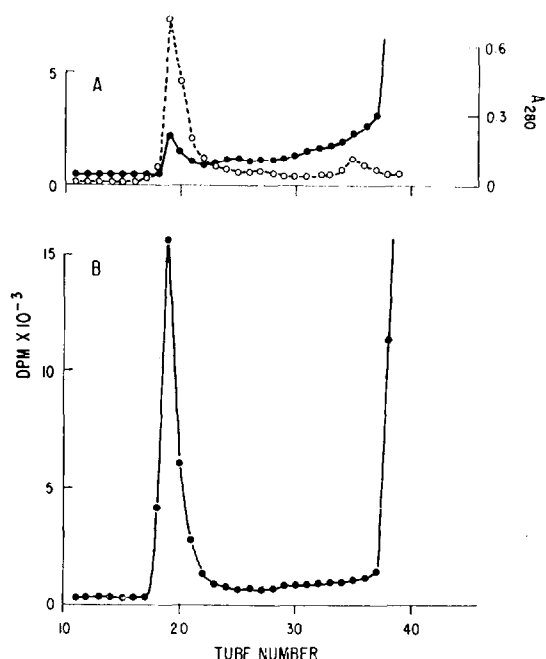
Fig. 1.—(A) Gel filtration of nuclease in 0.05 M Tris-HCl, pH 8.1, on a Sephadex G-25 column (2 × 40 cm).

(B) Gel filtration of nuclease after addition of calcium chloride and pdTp, using the same column equilibrated with the Tris-HCl buffer containing 0.01 M CaCl$_2$ and 6 × 10^{-4} M pdTp.

Optical density at 280 mμ (- -O- -O- -) and disintegrations per minute (—●—●—) are plotted.

has been shed from the protein during its passage through the column. Figure 1B gives the elution pattern of an identical aliquot of nuclease to which calcium chloride and pdTp were added ten minutes before application to the Sephadex column. The Tris-HCl buffer on the column contained these two ligands at the indicated concentrations. In contrast to the results summarized in Figure 1A, much more of the radioactivity was now associated with the protein peak and the amount back-exchanged during filtration was lower. The radioactivity in the protein-containing fractions corresponded to about 35 H^3 atoms per molecule of protein.

The relative difference between the free and liganded proteins depends on the duration of the gel filtration. Figure 2A shows the radioactivity pattern of the gel filtration of an aliquot of nuclease on a 2 × 15-cm Sephadex G-25 column. The summed disintegrations per minute are listed in Table 1. In four separate experiments this value averaged 17,300 (±3000). Figure 2B shows the elution pattern from the same column after addition of ligands to the aliquot of nuclease and to the column buffer. The trapping of tritium by the ligands occurred but the relative effect was smaller than in the first experiment because under these conditions there is less time for exchange-out. The trapping effect of the ligands must be very rapid compared to the separation occurring in the gel because a very similar pattern was obtained when nuclease, without ligands, was applied to a column which had been previously equilibrated with buffer containing both ligands (Fig. 2). On the other hand, omission of the ligands from the column buffer resulted in a substantial decrease in the radioactivity associated with nuclease to which ligands had previously been added. This was presumably due to dissociation of the protein-ligand complex occurring within the column.

TABLE 1. *Radioactivity associated with protein after gel filtration.*

Expt.	Protein solution	Ligands added	Σ dpm*	T/molecule*
1†	Nuclease	—	3,900	5
	Nuclease	Ca^{++}, pdTp	28,400	35
2	Nuclease	—	20,300	27
	Nuclease	Ca^{++}, pdTp	41,900	57
	Nuclease	Ca^{++}, pdTp‡	36,600	50
3	Nuclease	—	14,600	18
	Nuclease	Ca^{++}, pdTp	37,800	46
	Nuclease, Ca^{++}, pdTp§	Ca^{++}, pdTp	16,800	20
4	Nuclease, EDTA	—	9,200	13
	Nuclease, EDTA	Ca^{++}	19,000	27
	Nuclease, EDTA	pdTp	17,600	30
5	Nase-T-p$_2$	—	0	0
	Nase-T-p$_3$	—	0	0
	Nase-T-p$_2$	Ca^{++}, pdTp	300	<0.1
	Nase-T-p$_3$	Ca^{++}, pdTp	200	<0.1
	Nase-T-p$_2$, —p$_3$	—	3,800	3
	Nase-T-p$_2$, —p$_3$	Ca^{++}, pdTp	21,400	18

* The sum of disintegrations per minute associated with the protein peak and the gram atoms of tritium per mole of protein is calculated as described in *Methods*.

† A 40-cm column rather than 15 cm as in other experiments.

‡ Ligands were added to column buffer only.

§ Ligands were first added to nuclease before the addition of THO, again 10 min before application to the column, and to the column buffer.

Inhibition of ligand effect: If calcium chloride and pdTp were added to the nuclease solution before labeling with THO, trapping of tritium by the further addition of these ligands to the radioactive protein did not occur, as illustrated in Figure 3 and Table 1. In terms of our analogy, it is difficult to pour water into a corked bottle. These results also exclude the possibility that the ligands themselves contribute significantly to the increase of radioactivity associated with the protein complex.

Effect of calcium chloride or pdTp alone: Preliminary gel filtration studies showed that calcium chloride alone increased the binding of tritium to nuclease very slightly. However, pdTp alone gave most of the effect of both ligands in combination. With nuclease previously treated with ethylenediaminetetraacetate (EDTA) (final concentration of 0.01 *M*), different results were obtained. As summarized in Table 1, each of the ligands alone did cause some trapping of tritium, but not the full effect that was seen with the ligands in combination. A full understanding of the effects of ligands, individually, will require further study.

Tritium binding by nuclease-T fragments: We have applied the method described above to Nase-T-p$_2$ and Nase-T-p$_3$ and to nuclease-T in the presence and absence of calcium ions and pdTp.

Gel filtration of the separated, tritium-labeled Nase-T-p$_2$ and Nase-T-p$_3$ showed no radioactivity over background (Fig. 4*B* and *C*). When identical samples of labeled Nase-T-p$_2$ and Nase-T-p$_3$ were mixed before application to the column, there was a significant increase in bound radioactivity, as shown in Figure 4*A*. Of interest was the high level of radioactivity following the polypeptide peak, denoting tritium atoms with back-exchange rates of minutes.
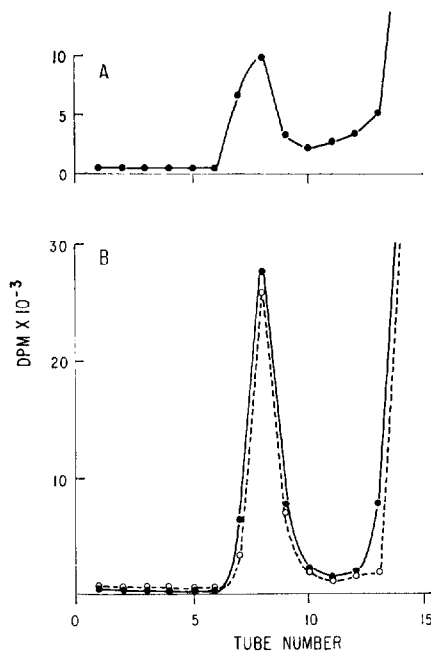
Fig. 2.—(A) Gel filtration of nuclease in 0.05 M Tris-HCl, pH 8.1, on a Sephadex G-25 column (2 × 15 cm). (B) Gel filtration of nuclease after addition of calcium chloride and pdTp (—●—●—) or without previous addition of the ligands (- -O- -O- -); both using the same column equilibrated with the Tris-HCl buffer containing 0.01 M CaCl₂ and 6 × 10⁻⁴ M pdTp. All lines indicate disintegrations per minute.

As with intact nuclease, the addition of ligands resulted in a dramatic increase of bound tritium in the nuclease-T (Fig. 4D). It is apparent that the exchange rates of a large number of tritium atoms have been appreciably slowed. The radioactivity associated with the single Nase-T-p₂ and Nase-T-p₃ after the addition of these two ligands, listed in Table 1, was insignificantly above background.

Discussion.—The study of hydrogen exchange in macromolecules has potential for describing dynamic aspects of the behavior of molecules in solution, but present theory and available experimental data do not yet allow a complete interpretation of the kinetics of hydrogen exchange with respect to the details of macromolecular conformation.[2,18] However, effects on the kinetics of hydrogen exchange have been reported for a number of protein-ligand systems,[5-9] and have been interpreted as evidence for induced conformational changes in the protein components. DeLuca and Marsh[7] found that the addition of substrates to luciferase, after separation from free THO, decreased the exchange-out rate of many hydrogens.

In our studies of such interactions, proteins were tritiated in the "relaxed" state and then separated from free THO by gel filtration at relatively low flow rate. Radioactivity associated with the protein peak thus represents tritium atoms with "intermediate" or "slow" exchange rates (see refs. 2 and 4 for the classifications); the tritium atoms in "instantaneous" and "fast" classes exchange-out during the 15 minutes or more required for gel filtration. The interactions studied here result in a marked increase in the class of measurably exchanging hydrogens. Thus, under conditions of defined temperature, ionic strength, pH, and time, the specific radioactivity of one protein peak is a useful parameter of molecular associations.

The very few tritium atoms in unliganded nuclease (Table 1) suggest that most of the labile hydrogens are accessible to solvent, presumably the result of relatively loose folding or of great motility[13] in the protein. The addition of calcium chloride and pdTp markedly increased the number of tritium atoms in the "intermediate" and "slow" exchanging classes. This could be caused by a shielding of part of the protein molecule from the solvent by the ligands, by a decrease in motility, or by a combination of these factors. Although other

FIG. 3.—(*A*) Gel filtration of nuclease in 0.05 *M* Tris-HCl, pH 8.1, on a Sephadex G-25 column (2 × 15 cm). (*B*) Gel filtration of nuclease after addition of calcium chloride and pdTp (—●—●—), and nuclease to which these ligands had been added before labeling and at the time of gel filtration (—·□—·□·); both run on the same column, equilibrated with the Tris-HCl buffer containing 0.01 *M* CaCl$_2$ and 6 × 10^{-4} *M* pdTp. In (*A*), optical density at 280 mμ (- -O- -O- -) and disintegrations per minute (—●—●—) are indicated; in (*B*), both lines refer to disintegrations per minute.
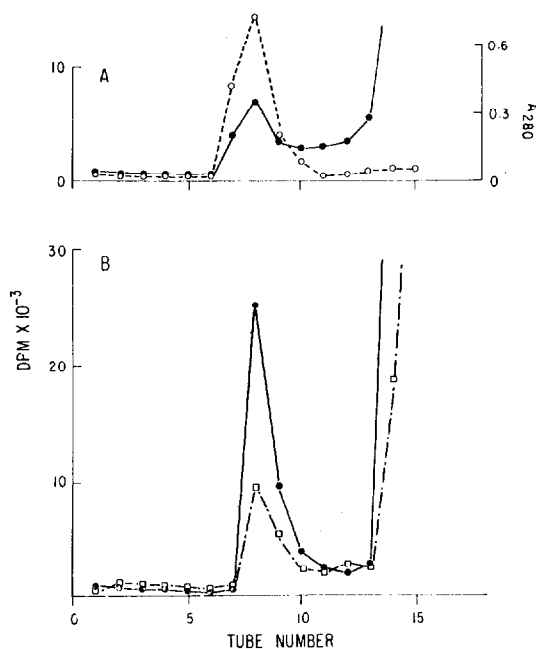
studies have suggested that the major effect of these ligands is expressed in a crevice region, they might not have detected a ligand-induced change in motility.

The addition of ligands before the addition of THO prevented an increase in tritium binding upon further addition of ligands to the labeled complex. This can be explained by assuming that during the time of this experiment the presence of the ligands excluded from labeling those hydrogens responsible for the differences seen during the trapping phenomenon.

The effects on tritium exchange of calcium chloride and pdTp used separately are most easily explained on the assumption, in accord with the experience of others,[19] that some calcium is ordinarily bound to the protein preparations. After EDTA treatment of nuclease, the accessibility of the protein's hydrogens to the solvent is increased. Addition of calcium chloride or pdTp alone increased the bound radioactivity but to a much lower extent than with the ligands in combination.

The absence of tritium atoms associated with the gel-filtered Nase-T-p$_2$ and Nase-T-p$_3$ indicates the ready accessibility of all of the exchangeable hydrogens to solvent. Complete exchange-out would be expected in a random-coil polypeptide.[2] The fact that tritium remains with the mixed fragments after they are gel-filtered is physical evidence for their interaction in solution and the establishment of a solvent-excluding conformation. This finding is compatible with the restoration of partial enzyme activity and changes in optical rotatory dispersion[12] and circular dichroism[20] measurements upon mixing the fragments. The absence of tritium trapping after the addition of the ligands to the individual fragments suggests weak or no binding but does not exclude the possibility of binding of these ligands unaccompanied by changes in structure or motility. Conversely,
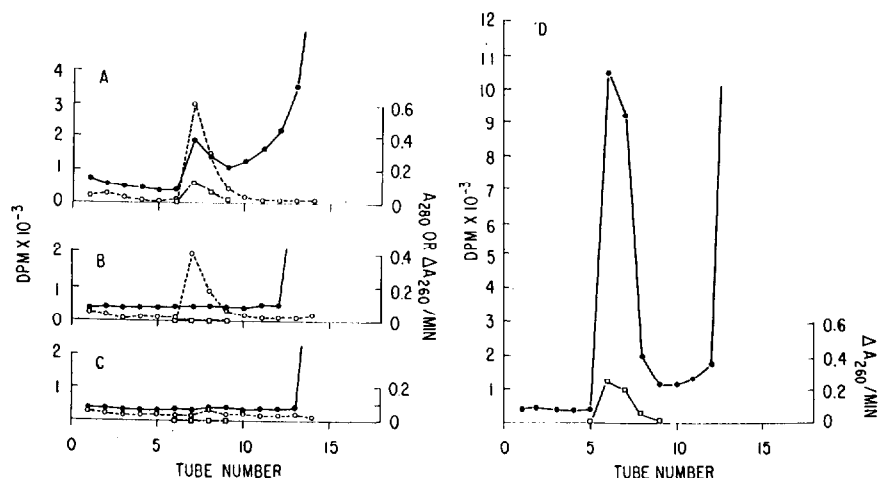
Fig. 4.—(*A*) Gel filtration of the equimolar mixture of Nase-T-p₂ and Nase-T-p₃ on a Sephadex G-25 column (2 × 15 cm), in 0.05 *M* Tris-HCl, pH 8.1.

(*B*) and (*C*) represent the gel filtration of 2.3 mg of Nase-T-p₃ and 1 mg of Nase-T-p₂, respectively, on the same column and in the same buffer.

(*D*) shows the results of the gel filtration of the equimolar mixture of Nase-T-p₂ and Nase-T-p₃ to which calcium chloride and pdTp had been added before application to the same column, previously equilibrated with the Tris-HCl buffer containing 0.01 *M* CaCl₂ and 6 × 10⁻⁴ *M* pdTp.

Optical density at 280 mμ (- -○- -○- -), disintegrations per minute (—●—●—), and change in absorbancy at 260 mμ in nuclease assay[16] (-·□—·□·) are indicated.

when ligands are added to a mixture of Nase-T-p₂ and Nase-T-p₃, there is a sixfold increase in the trapping of tritium in comparison to that seen with the mixed fragments without ligands. The amount of bound tritium in the nuclease-T preparations is less than that in intact nuclease, suggesting that the covalent change has made the structures more accessible to solvent.

Since gel filtration methods exclude the determination of the "instantaneously" exchanging hydrogens, the advantage of labeling in a relaxed state and then measuring the tritium trapped (at zero time of exchange-out) in the ligand-stabilized form should be noted. Thus, molecules may be labeled to different degrees at zero time of exchange-out but the kinetics of their *measurably* exchanging hydrogens may be very similar. Such effects could explain our findings, using the interrupted-flow gel filtration technique, that the sizes of the several classes of exchangeable hydrogens in the conformationally dissimilar[21] myoglobin and apomyoglobin are similar, yet the addition of hematin to the labeled apoprotein causes tritium trapping.[22] The ligand effects shown in Figure 3 also illustrate these points.

Under the standard conditions of our experiments, the average trapping caused by calcium chloride and pdTp raises the number of tritium atoms bound per molecule of nuclease from about 23 in the free form to about 52 in the presence of the ligands. It is unlikely that this difference results solely from stabilization of helices. The helix content as determined by optical rotation and circular dichroism, which does not change with the addition of ligands, would account for

only about 27 slowly exchanging hydrogens.[10] The direct shielding of the molecule from solvent, or a change in motility caused by ligands, must thus be considered.

Summary.—The addition of the ligands, calcium chloride and deoxythymidine-3',5'-diphosphate, to a staphylococcal nuclease previously labeled with tritiated water resulted in a marked decrease in the exchange-out rate of some tritium atoms. The mixing of two associating polypeptide fragments derived from this protein resulted in binding of tritium that was not observed in the separated fragments. The trapping of tritium demonstrated by this method may be a sensitive and simple probe for various interactions of macromolecules.

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